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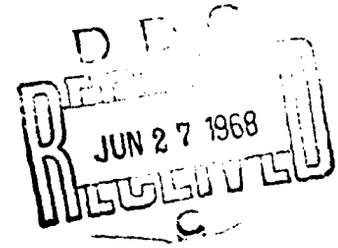

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ANALYSIS OF THE CHARACTERISTICS OF IMMUNE SERA IN RELATION TO THEIR LABELING WITH FLUOROCHROMES

Boll. Soc. Ital. Biol. Sperim. (Bulletin of the Italian Society of Experimental Biology), Vol. 39, 1963, pp 1562-1584.

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Institute of Normal Human Anatomy, Histology and General Embryology, University of Genoa

The introduction of fluorescent radicals into the protein molecules of a serum leads to a modification of their physical-chemical properties due to the blockade of the free NH_2 groups, which the fluorochrome accomplishes by means of a carbamide linkage (fluorescein isothiocyanate; FITC) or sulfamide linkage ("sulforodamina," 1-dimethylamino-t-naphthalenesulfonyl chloride, DANS) [1]. As a result, there occurs a lowering of the isoelectric point of the protein with a resulting relative acidification of the serum; this phenomenon plays an important role in the mechanism of aspecific reactions in immunohistological technique [1, 2]. The processes required for the labeling comprise, in addition, a series of inevitable technical manipulations which are capable of leading to modifications of the antibody spectrum of the serum and, more generally, to a decrease of its protein content. Only the sera are labeled when adding a quantity of fluorochrome equal to 1-5% of the total protein content, then filtering through dextran gel and again concentrating. During these phases of preparation a loss of protein is inevitable; this has been estimated at about 20% [3]. However, there are still no precise data available on the variation of the antibody content of a serum as a result of its labeling, nor on any modifications of the behavior of the conjugated antibodies in certain immunochemical tests.

For this reason we decided to test the behavior of a group of anti-human serum of rabbit sera (globulin fractions) during the different phases of preparation of the fluorescent conjugates.

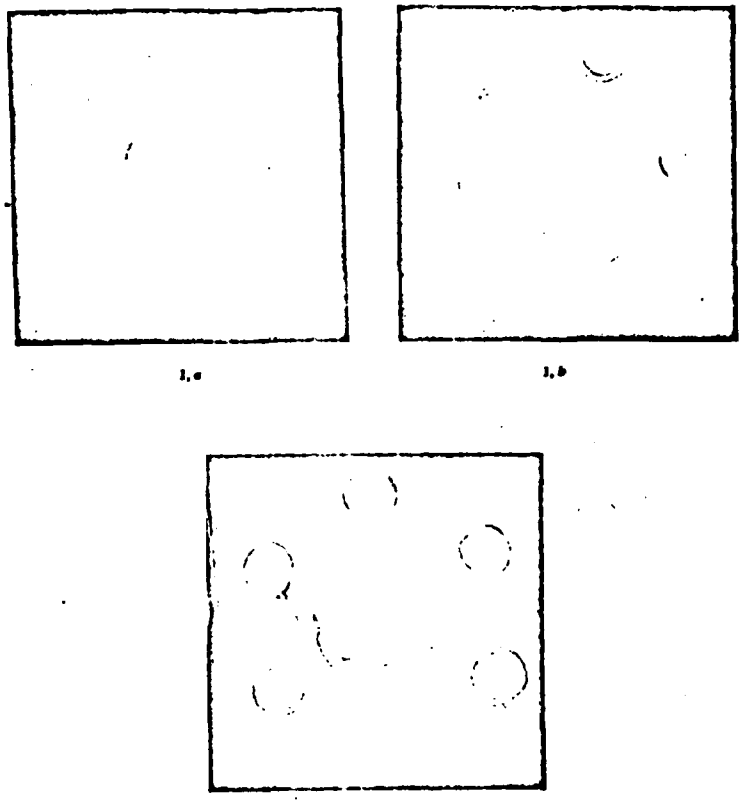

Fig. 1. Ouchterlony's Test.

a) Anti-human serum rabbit serum; b) Same after conjugation with DANS; c) Same after conjugation with FITC; Conjugates show a decrease of the number of precipitation lines.

For this study we made use of the α-precipitation test, ~~of~~ the techniques of two-dimensional immunodiffusion according to Ouchterlony, ~~[4]~~, and ~~of the~~ immunoelectrophoretic analysis on agar gel according to Scheidegger's micromethod [5, 6]. In all phases of the preparation of the sera we have, in addition, carried out -- still on agar gel -- an electrophoretic separation of the various protein constituents,

The fluorochromes used for this study were commercially available FITC, and DANS prepared by ourselves from 1-aminonaphthalene-5-sulfonic acid (Laurent's acid) according to Mayersbach [7].

For labeling with FITC we adhered to the technique of Riggs et al. [8] and Marshall et al. [9], adding the fluorochrome to the serum in powder form; the DANS was added to the serum in 1% acetonic solution.

The α-precipitation- and immunodiffusion tests were carried out either on native sera or after the addition of fluorochrome and passage through Sephadex G 25 Medium with subsequent concentration in Aquax (Gurr, London).

The protein content of the sera was determined by the method of Folin-Ciocalteau [10].

Finally we tried to evaluate the effects of the adsorption of serum on liver powder, since notable changes took place in the latter following this treatment [11]. To this end we used the powdered liver of guinea-pigs, treated with acetone according to the method of Coons [12], and added at the rate of 100 mg/ml of serum.

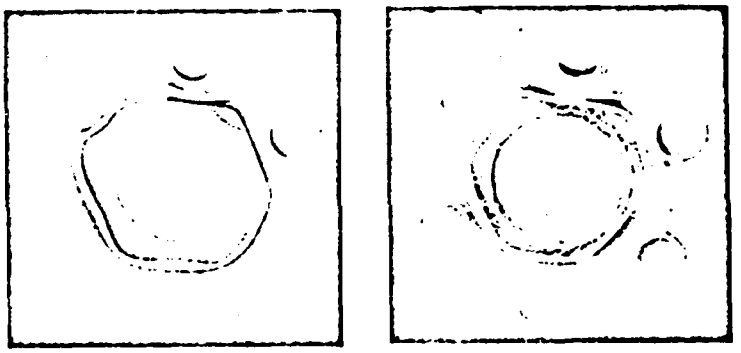

Fig. 2. Ouchterlony's Test.

a) Anti-human serum rabbit serum; b) Same after conjugation with DANS.

The total protein content of the sera was found to have decreased, as a result of labeling, by an amount varying between 20 and 35% of the initial value. This loss of protein took place during the passage through Sephadex.

The α-precipitation test, in certain cases prematurely positive and of a high titer, has shown differences in the behavior of the conjugated sera. While, in effect, in the native sera the appearance of an immune precipitate could be noted already after 1-2 hours at 37°C, this was not so in the case of

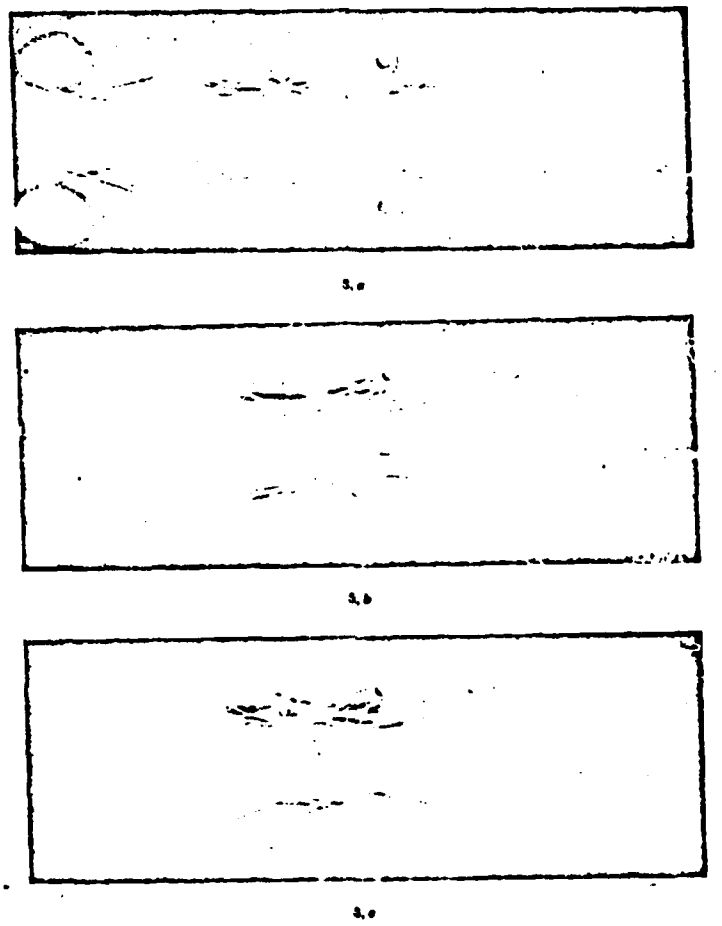

Fig. 3. Immunoelectrophoretic Patterns.

a) Anti-human serum rabbit serum. Electrophoretic pattern of antigen superimposed; b) Same after conjugation with DANS. Note the total disappearance of the antialbumin and anti-α_1-globulin lines and the marked attenuation of the anti-γ-globulin line; c) Same after conjugation with FITC. Disappearance of the anti-albumin antibodies, considerable modification in the anti-α_1 complex and in the anti-γ-globulin line.

the conjugated sera, for which at least 24 hours at 4°C were necessary to obtain a positive reaction. Nevertheless no noteworthy modifications of the precipitating titer of the labeled sera was observed; after 7 days at 4°C, this was practically identical with that of the non-conjugated sera. This behavior seems to be common to both types of fluorochromes employed, and did not seem to be affected by the presence of large amounts of free fluorochrome. In fact the results have been practically identical both for the sera filtered through Sephadex and for those tested directly after the addition of the fluorochrome.

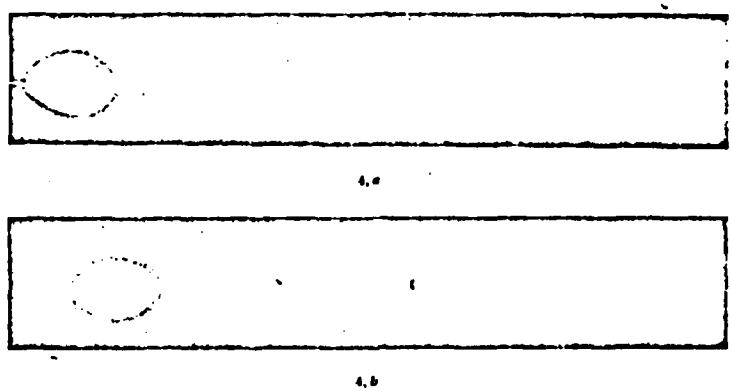

Fig. 4. a) Electrophoretic pattern of a human serum;
b) Same adsorbed once on powdered guinea-pig liver.

By means of the Ouchterlony test and the immunoelectrophoresis test we were able to learn that the decrease of the protein content of the sera sometimes corresponds to a loss of antibodies. This phenomenon may take place during two phases of the labeling, viz,

1) as a result of a denaturing of the protein through the action of the fluorochrome or, in the case of DANS, also of its solvent (acetone);
2) as a result of the stopping of the protein chains in the Sephadex column.

We have been able to ascertain that only a negligible part of the serum protein is denatured by the addition of fluorochrome. In fact the number and characteristics of the precipitation lines identified on Ouchterlony's slides or in the immunoelectrophoretic patterns are practically unchanged after the addition of the fluorescent dye. On the other hand, after filtration through Sephadex and concentration in Aquax, we note a decrease in the number of these precipitation lines (Figs. 1 and 2).

The immunoelectrophoretic analysis has enabled us to demonstrate in certain cases the selective and complete disappearance of the anti-albumin antibodies, as well as a notable decrease of the antibody-globulin ratio (Fig. 3). The anti-α_2 and anti-β globulin antibodies, were relatively well preserved, while above all the number of anti-α_1 and anti-γ-globulin antibodies decreased (Fig. 3).

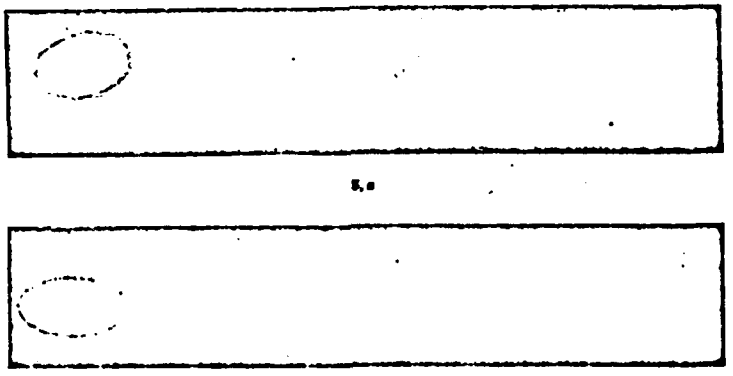

Fig. 5. a) Electrophoresis of rabbit serum; b) Same after conjugation with DANS. Under the same conditions (time and voltage) there may be observed a greater migration of the negatively charged proteins toward the positive pole.

Treatment with liver powder has had a notable influence on the protein mixture of the sera, leading to the decrease or disappearance of α_1-globulin (Fig. 4). Hence the data of Mayersbach [11] are confirmed.

The lowering of the isoelectric point of the conjugated seroproteins, or rather the relative prevalence of negative charges, explains why a conjugated serum may be fractionated by electrophoresis in a shorter time than native serum (Fig. 5), and is perhaps also able to explain the marked delay which we observed in the appearance of a positive α-precipitation test.

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